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THE INVERTASE OF MYROTHECIUM VERRUCARIA SPORES[1]

G. R. Mandels

IN THE COURSE of studies on the germination and respiration of spores of *Myrothecium verrucaria* it was found that they had considerable invertase activity (Mandels and Norton, 1948). The ease with which the conidia of this fungus can be raised and handled and the simplicity of measuring invertase provided a good experimental basis for an investigation of the behavior of an assumedly typical carbohydrase in the physiology of the spores. Invertase has been demonstrated to occur in the spores of several Aspergilli and Penicillia (Kopeloff and Kopeloff, 1919; von Euler, 1920–1921; Sumi, 1928), however, no detailed study of the enzyme in the spores has been encountered. This paper presents results of a study of the physiological relationships of the enzyme. Specifically it was desired to compare the action of the enzyme *in vitro* and *in vivo* to determine the locus of activity of the enzyme in the spores and the conditions under which synthesis and secretion occur.

METHODS.—The fungus used in these studies, *Myrothecium verrucaria* (Alb. and Schw.) Ditm. ex Fr. USDA 1334.2, has "masqueraded" under the name *Metarrhizium glutinosum* Pope for several years (White and Downing, 1947). Spores for experimental purposes were obtained by culturing the organism on filter paper (Whatman #2) in 250 ml. Erlenmeyer flasks containing 50 ml. of the inorganic nutrient solution (distilled H_2O — 1000 ml., NH_4NO_3 — 3.0 g.; $MgSO_4 \cdot 7H_2O$ — 2.22 g.; KH_2PO_4 — 2.59 g.; K_2HPO_4 — 2.21 g.; *p*H *ca* 6.5) to which 1.5 per cent agar (Bacto) had been added. These flasks were inoculated with 1 ml. of an unwashed suspension of spores in distilled wa-

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ter (spore concentration *ca* 500/mm^3) and placed in an incubator at 30°C. Under these conditions spore formation is initiated after about 3 days and is completed about 4-5 days after inoculation. This method provides spores of approximately the same age. This is in contrast to the results obtained by using a point inoculation in the center of the culture in which case sporulation extends over a period of several weeks. The age of the spores obtained using this surface inoculation method can thus be approximated by subtracting 4 days from the age of the culture. Some caution must be exercised in using this method of inoculation, since it has been found that this organism produces occasional spontaneous variations. To decrease the possibility of variant strains contaminating the cultures, flasks inoculated in the center with a needle were always used to prepare the suspensions which served as inoculum for the stock cultures. By this procedure it was ascertained that the inoculum had all come from a monotypic culture since sectors of variants would be discernable. Contamination by variant spores is believed to be insignificant.

Spores for experimental purposes were harvested by flooding the surface of the culture with buffer or distilled water and shaking gently for a few moments. Since the spores are borne in a mass of slime, suspensions are prepared easily without the use of any wetting agents. The spores were washed three times by repeated centrifugation and decantation. The concentration of spores in suspensions was determined by measuring the transmission with a photoelectric colorimeter, using a filter having a transmission maximum at 440 $m\mu$.

Invertase activity was measured by incubating the spores in solutions of sucrose (J. T. Baker's C.P. crystals) and determining the reducing sugars formed. The dinitrosalicylic acid method of Sumner was used. (Sumner and Somers, 1944). When determinations were made using spore suspensions the spores were removed by centrifugation. Generally an aliquot of sodium hydroxide solution was added before centrifuging to stop the enzyme action. In a few experiments where the sensitivity of the dinitrosalicylic acid method was inadequate, the method of Folin and Malmros (1929) was employed. Invertase activity is expressed in terms of milligrams of reducing sugars (as glucose) formed per hour. In some instances this is on a basis of unit volume of suspension, in others on a basis of 10^8 or 10^9 spores. During incubation of spores in sucrose solutions, the containers were shaken on a reciprocal shaker at 30°C. or on a rotary shaker at room temperature.

RESULTS.—The rate of hydrolysis of sucrose by the spores is directly proportional to the spore concentration over the range of 2360-39,000 spores/μl. (table 1). All of the experimental work was done within this range of spore concentration.

Determination of the activity of spores from cultures of different ages (spore age = culture age − 4) indicates a slight increase in activity as the spores age (table 2). It is possible that this effect is apparent rather than real, since the light transmission of supensions of equal numbers of spores may vary with the age of the spores and the calibration curve used to determine spore concentration was established with spores of one age only (11 days). This effect should not be very great, however, and it can be concluded that invertase activity does not change very markedly over the range of spore ages used.

TABLE 1. *Effect of spore concentration on rate of hydrolysis of sucrose.*[a]

Spore conc. (final)	Mg. red. sugars /hr./ml.	Mg. red. sugars per hr./10^8 spores
39,000/mm^3	0.32	0.82
19,500	0.183	0.94
8,960	0.072	0.80
4,800	0.038	0.79
2,360	0.025	1.06

[a] 0.01M KH_2PO_4; 8% sucrose; spores from 20-day-old culture.

To determine whether the substrate upon which the spores were raised had any influence on their invertase activity, a series of flasks was prepared containing nutrient agar to which different carbon sources were added. Inoculation was with a suspension of washed spores. The substrates used were: glycerine, xylose, glucose, fructose, mannose, sucrose, cellobiose, maltose, starch, cellulose (filter paper) glutamic acid (adjusted to *p*H 6.5), casein hydrolysate (acid hydrolyzed), peptone, potato dextrose agar (Difco). When the cultures were 11 days old sporulation had occurred in all the cultures which contained carbohydrates. Only sparse spore formation occurred in the potato dextrose agar culture. Those having glutamic acid, casein hydrolysate, or peptone grew but did not sporulate. Spores were removed from the cultures which sporulated, washed, suspended in buffer, and the invertase activity determined. The data (table 3) show that while slight differences occur, the spores have substantially the same activity regardless of the substrate upon which they were raised.

TABLE 2. *Effect of spore age on invertase activity.*[a]

Culture age	Red. sugars/hr./10^9 spores
8 days	5.7 mg.
15	6.1
22	6.6
33	6.7
46	7.3

[a] Inorganic nutrient solution *p*H 4.5; 5% sucrose; room temp. = 25° ± 1°C.

TABLE 3. *Invertase activity of spores raised on different substrates.*[a]

Carbon source	Mg. red. sugars/hr./10^9 spores
Glycerine	4.4
Xylose	4.3
Glucose	4.1
Fructose	3.7
Mannose	4.0
Sucrose	4.7
Cellobiose	4.1
Maltose	3.9
Cellulose	3.6
Starch	4.0

[a] 0.01M KH_2PO_4; 5% sucrose; spores from 11-day-old cultures.

Course of the reaction.—Early in the experimental work the question arose as to the existence of a lag in the hydrolysis of sucrose by spore suspensions. The data shown in fig. 1 (obtained by using the method of Folin and Malmros) indicate a lag in hydrolysis of very short duration, being of the order of 1 min. The reducing value at zero time represents the impurities in the sucrose used. For the present purposes this lag can be disregarded although the theoretical implications may be significant.

The subsequent course of the reaction over a period of 6 hr. was determined in nutrient solution at *p*H 4.5 as well as in phosphate buffer. Yeast extract (0.5 per cent) was added to some of the flasks which brought the *p*H to between 5 and 6. The results are shown in fig. 2. Without yeast extract in phosphate buffer the invertase activity is essentially constant. In nutrient solution the rate is slightly greater than in buffer and is constant for 4-5 hr. By 6 hr., however, the rate has increased slightly. Where yeast extract was added the rate of hydrolysis increases rapidly after 2 hr. No significant differences are observed between the buffer and the nutrient solution. While no appreciable growth occurs over this 6 hr. period with sucrose alone, addition of yeast extract results in growth (*i.e.*, swelling of spores, increase in dry weight) and germination occurs. This increase in activity represents the synthesis of invertase which would be expected to accompany growth. Further consideration of the relation between synthesis and growth will be dealt with in a subsequent paper.

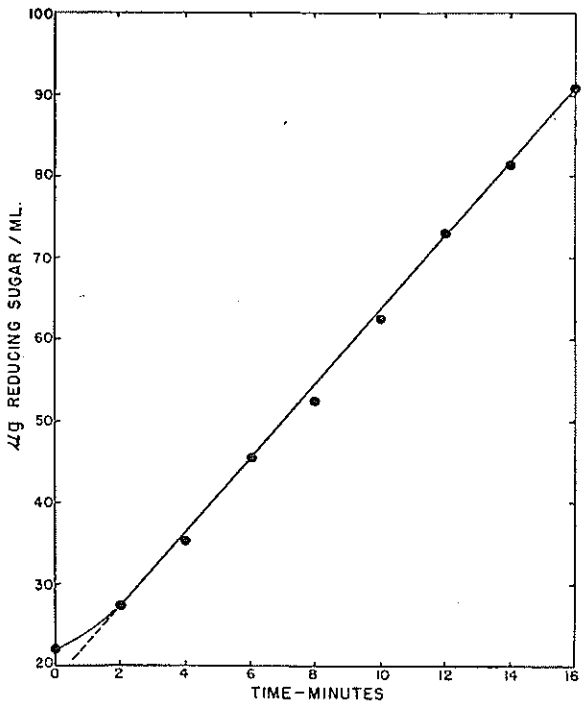

Fig. 1. Initial course of hydrolysis of sucrose by spores.

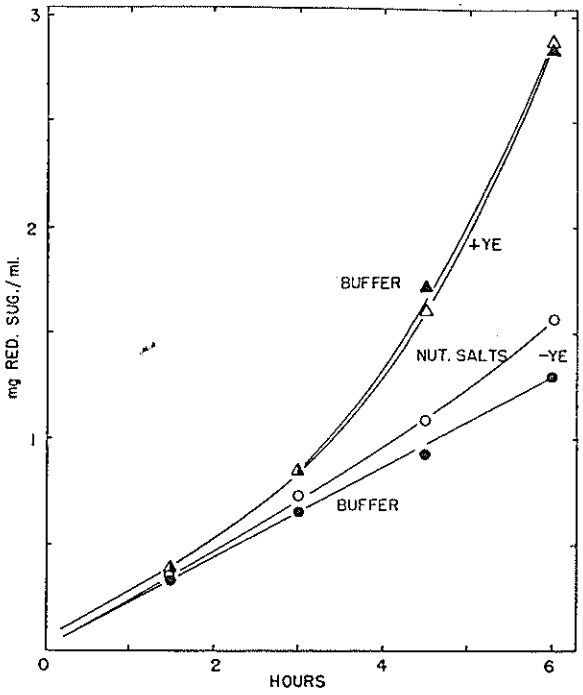

Fig. 2. Course of sucrose hydrolysis by spores.

Assimilation of sugar during hydrolysis.—The increase in dry weight of young spores (from 9-day-old cultures) suspended in nutrient solution at *p*H 5.1 containing 2 per cent sucrose was determined, as well as the formation of reducing sugars. The data (table 4) show that assimilation does occur, the increase in dry weight being equal to about 8 per cent of the reducing sugars formed. If this assimilation is at the expense of reducing sugars, the measured invertase activity would be significantly lower than the actual rate.

In a similar series to which 10^{-3} M sodium azide was added no assimilation occurred. Furthermore

TABLE 4. *Assimilation of sugar during hydrolysis.*[a]

Treatment	Time	Dry wt. spores/ml. susp.	Dry wt. increase	Red sugars ml.
	0 hr.	1.28 mg.	0 mg./ml.	0.0 mg.
Control	3	1.41	0.13	1.6
	5.5	1.54	0.26	3.3
	0	1.27	0	0.0
10^{-3} M	3	1.20	0	1.4
NaN_3	5.5	1.22	0	2.2

[a] Inorganic nutrient solution *p*H 5.1; 2% sucrose; spores from 9-day-old cultures; 30°C.

there was no increase in invertase activity as would be expected if reducing sugars were assimilated. It appears that direct assimilation of sucrose occurs (Hestrin, 1948). The more rapid hydrolysis of sucrose in the absence of azide is ascribed to synthesis of invertase.

Release of invertase from intact cells.—Preliminary experiments had shown that invertase was not released from the spores during a 16-hr.-period of shaking in inorganic nutrient solution. It was considerer possible that the enzyme was adsorbed to the spore walls. If this were true, then elution might be effected by suspending the spores in solutions of different *p*H. Neuberg and Roberts (1946) note that invertase adsorbed on aluminum hydroxide is quantitatively eluted by dilute alkaline solutions such as Na_2HPO_4. To test this possibility an experiment was set up in which spores were suspended in 0.1 M HCl (*p*H 1.2); 0.1 M H_3PO_4 (*p*H 1.7); 0.1 M KH_2PO_4 (*p*H 4.5); 0.1 M K_2HPO_4 (*p*H 8.7); 0.1 M Na_3PO_4 (*p*H 12.1). After shaking in these buffer solutions overnight, the spores were removed by filtration. The spore-free filtrates were tested for invertase activity by adding an equal volume of sucrose solution after adjusting the reaction of the filtrates to *p*H 4.5-5.5. No activity was found in any of the filtrates. Interpretation of this experiment cannot be conclusive as inactivation of any released enzyme may have occurred in the extremes of *p*H used. It has been found subsequently that while inactivation would have occurred at the two lowest *p*H levels (1.2, 1.7), under the conditions of the experiment some reactivation would have taken place.

To determine whether the differential permeability of the plasma membrane prevented the outward movement of the enzyme, merthiolate (0.01 per cent), toluene, or ether was added to spore suspensions. After incubation for about 16 hr., invertase activity of the spores and of the suspension medium was determined. No evidence was found that invertase was released from the spores and no inactivation of the invertase in the spores had occurred. The treatments killed over 95 per cent of the spores (based on 5 per cent survival). Thus destruction of the plasma membrane does not cause release of enzyme. It has been found that secretion of invertase (Mandels, 1949) occurs during growth when synthesis of the enzyme takes place. Possibly changes in permeability which permit diffusion of the enzyme out of the spores might result from or accompany the swelling and stretching of the cell wall and plasma membranes. An experiment was set up to test this hypothesis and to determine whether any of the invertase synthesized and retained by germinated spores can be leached out. Aliquots of a spore suspension in inorganic nutrient solution to which sucrose, sucrose + yeast extract or yeast extract was added, were incubated at 30°C. for 6 hr. The spores were then washed by centrifugation and suspended in buffer (0.01 M KH_2PO_4). These suspensions were divided into two equal parts to one of which merthiolate was added (to give 0.02 per cent). After incubating over night the suspensions were centrifuged, the supernatant decanted through a fine sintered glass funnel, and the invertase activity of spores and filtrate determined. The data are summarized in table 5. The activity of the spores in nutrient solution did not change from the original activity of the suspension which was determined separately. Considerable invertase was synthesized during incubation of the spores with the various substrates in the preleaching stages, the amount being greatest in the suspension containing both yeast extract and sucrose. None of the invertase was leached from the cells during the 17 hr. period in buffer, except where both yeast extract and sucrose were present, in which case 4-5 per cent of the total activity was found in the filtrate. It is possible that traces of invertase were released in the other instances. If a similar proportion of the total activity had gone into the filtrates, it would not have been detected by the methods employed. The addition of merthiolate, which killed most of the cells, did not have any effect on the release of enzyme.

Incubation of germinating spores with buffers at different *p*H's has also failed to effect release of invertase, as shown by data obtained in conjunction with the experiment described above in which

TABLE 5. *Is invertase released from germinated spores?*

Treatment	Germination	Merthiolate	Invertase activity[a] Spores	Filtrate
Control	—	+	0.26	0
		—	0.26	0
Sucrose	—	+	0.40	0
		—	0.37	0
Sucrose + yeast extract	++	+	1.17	0.06
		—	1.20	0.05
Yeast extract	+	+	0.44	0
		—	0.46	0

[a] Mg. red. sugar/ml./hr.

TABLE 6. *Invertase activity of extracts of ground spores.*

Preparation	Invertase activity (mg. red. sugar/hr.)
A Supernatant	0.14
A–F Filtered supernatant	0.13
B 1st Washing	0.013
C 2nd Washing	<0.005 (not detectable)

it was attempted to elute the enzyme from dormant spores.

It can be concluded that invertase is not released from dormant or intact germinated spores and that killing the spores by such agents as merthiolate or toluene does not effect release.

Extraction of invertase from crushed spores.—Spores which had been washed in distilled water and lyophilized were ground in a mortar and pestle to which about three times their weight of powdered pyrex glass and a small amount of 0.1 M KH_2PO_4 had been added. Additional buffer was added and the ground spores centrifuged in a Serv-all superspeed centrifuge at about 20,000 G. for 20 min. The slightly turbid supernatant liquid (A) was decanted off and a portion of it, (A-F), filtered through a bacterial filter (Corning, ultra fine sintered glass) to remove all traces of cell fragments. The resulting filtrate was clear. To determine whether additional enzyme could be extracted the residue of spores, *etc.* in the centrifuge tube was washed with a second portion of buffer and centrifuged (B). A third extraction of the residue was made by suspending the residue in buffer and storing it overnight in a refrigerator and then centrifuging (C). The invertase activity of these extracts is shown in table 6.

The data demonstrate that by rupturing the cell walls, and the plasma membranes as well, invertase can be extracted from the spores and obtained in solution. Furthermore, the enzyme is not bound to any cell structures which can be removed by filtration through a bacterial filter. Repeated washing of the residue is without effect in extracting additional portions of the enzyme.

Another critical point in the extraction of the enzyme is whether the total activity of the preparation changes when the spores are ruptured. Data (table 7) show that there is no such change, the activity of the filtrate plus the residue being equal to the original activity. Although only about 25 per cent of the invertase was extracted in this experiment, 70 per cent of the activity has been obtained in filtrates of other experiments. The amount extracted appears to depend upon the proportion of the spores which are ruptured.

TABLE 7. *Invertase activity of residue and of extract in comparison with original activity of spore suspension.*

Preparation	Invertase Activity
Residue	0.56
Filtrate	0.185
	sum 0.745
Original (not ground)	0.74

Effect of pH in vitro and in vivo.—Preliminary experiments had indicated that the *p*H for optimum invertase activity of *M. verrucaria* spores was similar to that for the action of purified invertase. It was reasoned that if the *p*H activity curves for the *in vitro* and *in vivo* action of *M. verrucaria* invertase were similar, this would constitute evidence that the enzyme was situated at, or in close proximity to, the exterior of the cytoplasmic membrane of the spore. Here the enzyme would be subject to the direct action of the solution in which the spores were suspended. For this reason the invertase activity of spores and of extracts from ground spores was determined as a function of *p*H. For comparative purposes the effect of *p*H on some purified yeast invertase (Difco) was also determined. Data from three sets of experiments are presented (fig. 3). The curves from *p*H 2.4-5.5 were obtained using 0.05 M citric acid—0.1 M Na_2HPO_4 buffer; the curves from *p*H 4.5-8.0 with 0.033 M KH_2PO_4 — K_2HPO_4 buffer. The points for values below *p*H 3.5 are all corrected for the hydrolysis catalyzed by the hydrogen ion since significant inversion occurred at these low *p*H's, under the con-

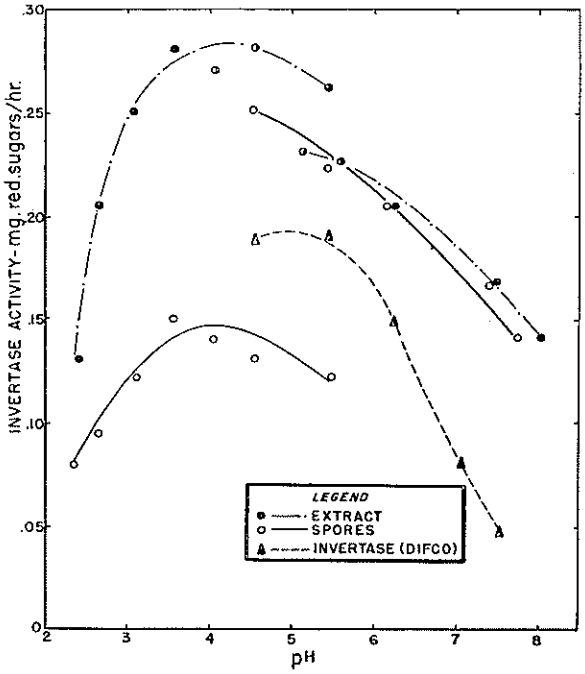

Fig. 3. Effect of *p*H on the invertase activity of *M. verrucaria* spores and of cell free extracts of the spores.

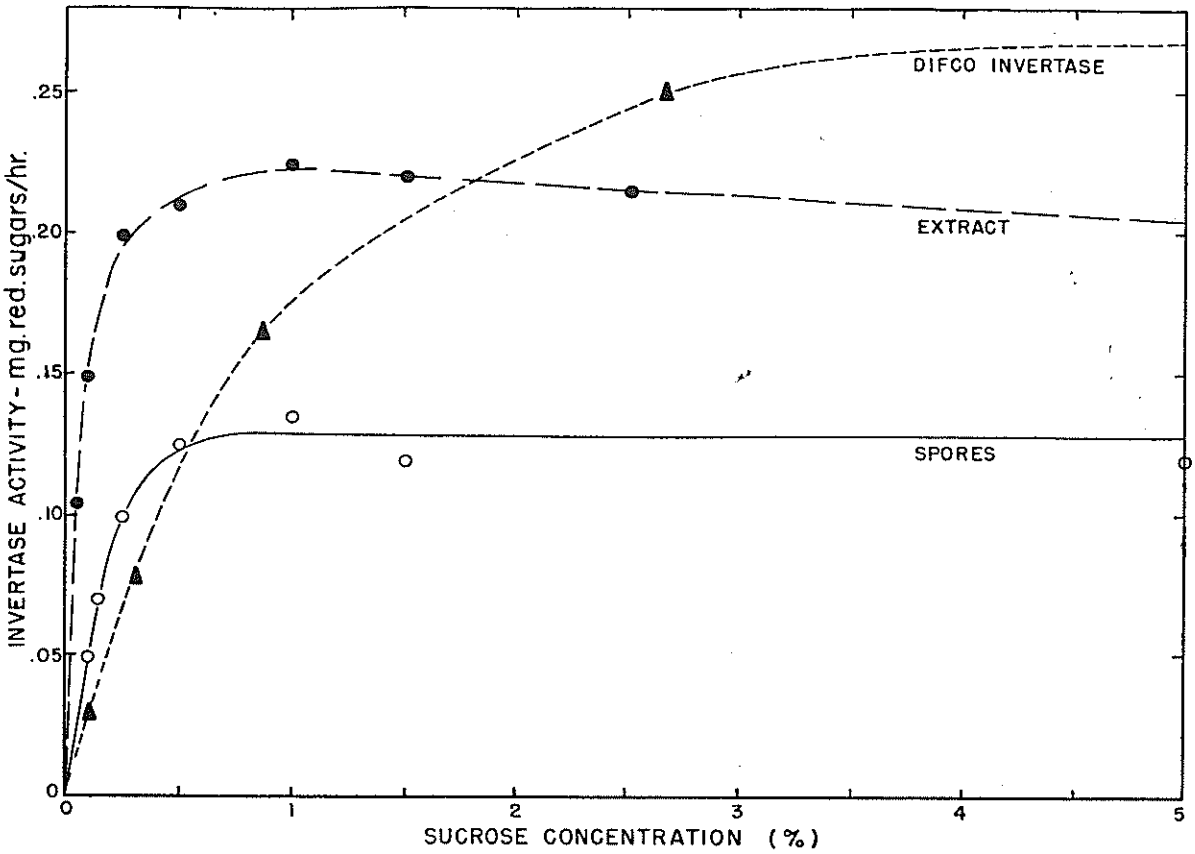

Fig. 4. Effect of substrate concentration on rate of hydrolysis of sucrose by spores, spore extracts, and Difco Invertase.

ditions employed. The curves for *in vitro* and *in vivo* action of invertase from *M. verrucaria* spores are similar, the p*H* optima being between 3.5 and 4.5. The curves are not exactly parallel since the optimum for the spore extract is displaced slightly toward the neutral side and the activity of the spore extract decreases more rapidly on the acid side of the optimum. The significance of these differences cannot be evaluated precisely. Possibly the optimum changes slightly when spores of different ages are used; possibly the presence of proteinaceous materials in the spore extracts modifies the optimum. In this connection it should be noted that copious precipitation occurred at the lower pH values in the spore extracts upon addition of the buffer. Since this precipitate settled to the bottom of the tubes this may be a complicating factor if the enzyme were present in the precipitate. The response of the purified yeast invertase is somewhat different from that of the *M. verrucaria* enzyme. The optimum of the former is displaced a little toward neutrality and its activity decreases more abruptly as the pH increases.

Effect of substrate concentration—in vitro and in vivo.—The relation between sucrose concentration and rate of hydrolysis by a spore suspension, an extract of ground spores and by purified yeast invertase (Difco) is shown in fig. 4. The curves are typical for velocity substrate concentration plots of enzymes such as invertase. The curves for the *in vivo* and *in vitro* action of the invertase from *M. verrucaria* spores are similar. The curve for the purified invertase, however, is somewhat different in that the change in rate of the reaction at low concentrations of substrate is less rapid than with the enzyme in the spores. Further characterization of the enzymes is shown in table 8 in which the Michaelis dissociation constants (K_S) for the enzyme-substrate complex are given. Two methods were used to calculate the dissociation constant. In the first method the substrate concentration at half maximum reaction velocity was calculated according to Michaelis and Menten (1913). For the second the procedure of Lineweaver and Burk (1934) was employed which is based on the equation

$$\frac{1}{V} = \frac{K_S}{V_{max}[S]} + \frac{1}{V_{max}}$$

According to this equation a linear relation should obtain if the reciprocal of the reaction velocity (V)

TABLE 8. *Dissociation constants for enzyme substrate complex.*

Preparation	Buffer	K_s Method a	K_s Method b
Spores (*ca* 20 days old)	0.01 M KH_2PO_4	0.0044 M	0.0047 M
" (26 days old)	0.01 M KH_2PO_4	0.0044	0.0035
" (28 days old)	0.025 M KH_2PO_4	0.0024	0.0022
" (27 days old)	0.01 M K_2HPO_4	0.0059	0.0056
Extract form spores 11 days old	0.025 M KH_2PO_4	0.0016	0.0017
Difco invertase (from yeast)	0.01 M KH_2PO_4	0.0175	0.025

(a) = Method of Michaelis and Menten (1913). (b) = Method of Lineweaver and Burk (1934).

is plotted against the reciprocal of the substrate concentration (S). The ordinate intercept is then the reciprocal of the maximum velocity ($V_{max.}$) and the slope is equal to $\frac{K_S}{V_{max.}}$. The equilibrium constant, K_S, is thus evaluated easily.

The dissociation constants obtained for the enzyme-substrate complex are distinctly less for the *M. verrucaria* invertase *in vitro* than *in vivo* although they are of the same order of magnitude. It is evident that the affinity of the enzyme in the spores for the substrate is less than that of the cell-free enzyme, affinity being inversely proportional to the dissociation constant. Possibly some of the reactive groups in the enzyme bind it to cell structures or are directed toward the interior of the cell and are not readily available to the substrate. Separation of the enzyme from the cell would make these groups available for combination with the substrate. The difference between the *in vitro* and *in vivo* values could also be interpreted on the basis of the permeability of the cell membrane limiting the diffusion of substrate to all portions of the enzyme. The value for yeast invertase by the first method is almost identical with that given by Michaelis and Menten. The difference between the values for Difco invertase and for the enzyme from the spores is of such magnitude as to indicate a difference in the enzymes; the enzyme from the spores having a greater affinity for the substrate. This is particularly evident in comparing the cell-free enzymes from *M. verrucaria* and from yeast.

DISCUSSION.—*Evaluation of effects of metabolism on measured and actual activity.*—Invertase activity of *M. verrucaria* spores is readily demonstrated by suspending them in sucrose solutions. The appearance of reducing sugars in the medium shows clearly that hydrolysis proceeds at a more rapid rate than utilization of reducing sugars in the metabolism of the spores. In presenting the data in this paper it has been tacitly assumed that the measured rate of hydrolysis does, in fact, represent the actual rate. It is quite possible that the measured rate is really a net rate equal to the difference between the actual rate of hydrolysis and the rate of utilization of reducing sugars by assimilation and respiration. The following discussion will attempt to evaluate these two possibilities.

Data (table 4) show that after 5.5 hr. incubation in sucrose solution the measured rate of hydrolysis is 0.60 mg. reducing sugar/hr./ml. and the rate of assimilation (dry weight increase) 0.047 mg./hr./ml. From other data (Mandels and Norton, 1948) we can approximate the amount of sugar oxidized as being about 0.053 mg./hr./ml. as calculated from the respiration of spores on sucrose. If it is assumed that assimilation and respiration are at the expense of reducing sugars then the actual amount of reducing sugar formed by hydrolysis of sucrose should be the sum of the measured quantity, the assimilated and the respired or about 0.70 mg./hr./ml. The actual invertase activity would then be *ca* 17 per cent higher than the measured rate. Treatment of the spores so as to block assimilation and respiration might be expected to increase the measured activity by this amount. In no case has this been observed. Such treatments have included the use of inhibitors (azide, cyanide, iodoacetate, fluoride), anaerobiosis, killing the spores by antiseptics, or heat treatment (60°C.). It is concluded that the actual and the measured invertase activity of *M. verrucaria* spores are identical for all practical purposes. It is not know whether these considerations are equally applicable to germinating spores and to mycelium.

This evidence is also suggestive that in the spores sucrose enters the metabolic pathway as such and need not first be hydrolized to glucose and fructose. No unequivocal evidence has been obtained proving this mechanism in *Myrothecium verrucaria.* Supporting indirect evidence is found in measurements of the rate of respiration of the spores on the carbohydrates involved. Thus the rate of respiration with sucrose as substrate is considerably greater than with either glucose or fructose alone and is slightly greater than with a mixture of these two reducing sugars (Mandels and Norton, 1948). While it would seem reasonable that metabolism of reducing sugars occurs coincidentally with that of sucrose, assuming that direct metabolism occurs, it is quite possible that this does not occur. Thus in the phenomenon of diauxie (Monod, 1947), preferential complete utilization of one carbohydrate occurs before a second carbohydrate is attacked.

Location of the enzyme.—Invertase is liberated from the spores in suspension only if growth and synthesis take place. In the resting spores hydrolysis of sucrose must occur either at the surface of the spore or within the spore. While it has not been possible to localize the site of invertase activity in the spores by direct demonstration, certain

indirect evidence has been encountered. Available data indicate quite clearly that invertase is not adsorbed to the spore wall. First, the enzyme is not eluted from intact spores suspended in solutions of varying *p*H under conditions which would quantitatively elute invertase adsorbed to aluminum hydroxide. Second, if the spores are crushed by grinding with powdered glass, the enzyme is obtained in solution. The activity of the slightly turbid supernatant solutions resulting from centrifugation at about 20,000 times gravity is not lost by passage through a bacterial filter which removes the turbidity. It is logical to assume that if the enzyme were adsorbed to the wall of the intact spores, this treatment would not elute it and it would remain adsorbed to the cell wall fragments and be thrown down by centrifugation.

It has been found that the *pH*-activity curves for suspensions of spores and for extracts of ground spores are closely parallel with an optimum at about *p*H 3.5-4.0. We assume that, as in the cells of higher plants, the cell wall is essentially completely permeable and that the *p*H within the spores is realatively constant and is not subject to the variations which occur in the environment. This suggests that the locus of invertase activity must be at, or in close proximity to, the exterior of the spore. Since the enzyme is not adsorbed to the spore wall we infer that it is at the external surface of the cell membrane. Possibly the enzyme is within the membrane but, if so, it must be sufficiently close to the external surface of the membrane to be subject to the direct action of the external solution. Additional data supporting this concept are to be found in the effects of low *p*H upon the invertase of the spores and upon spore viability. Thus exposure of spores to a *p*H of 1.5 is sufficient to inactivate the invertase without affecting the viability (unpublished data).

It may be argued that the factor limiting the rate of hydrolysis is the permeability of the spores to sucrose, and that the effect of *p*H upon the invertase activity of the spores can be ascribed to effects upon the permeability to sucrose. That such effects could coincide so closely with that of *p*H on the *in vitro* action of the enzyme is considered unlikely. It is concluded that the enzyme is not adsorbed to the spore wall but is present in the superficial layers of the cytoplasmic membrane or at its surface. Thus it is not necessary for sucrose to enter the spore to be hydrolyzed. The location of certain enzymes at the cell surface is not a new concept. It has been inferred for certain dehydrogenases in *Bacterium coli* (Quastel, 1926), for invertase in yeast (Wilkes and Palmer, 1932-1933) and for phosphatase in yeast (Rothstein and Meier, 1948).

Synthesis of the enzyme.—Measurements have shown that when spores are suspended in buffer or nutrient solution containing sucrose, there is no significant change in rate of hydrolysis until after about 4-6 hr., when slight increases are noted. Under these conditions no germination and little growth occur. If yeast extract is added as well as sucrose, growth occurs and the rate of hydrolysis increases rather rapidly. This increase in activity could be due to synthesis of invertase or to activation of preexisting enzyme. Since the rate of hydrolysis is not affected by substrate concentration at the levels employed, permeability is probably not involved. It is assumed that the observed increases in invertase activity are due to synthesis and that activation is not involved to any significant extent. This assumption is supported by the absence of any sizeable lag in inversion and by the failure of sucrose alone to effect activation. Furthermore, it is known that the metabolism of spores suspended in solutions containing sucrose and yeast extract is very high since large increases in cell volume, in dry weight and respiration are taking place. It is logical, therefore, to assume that synthesis of invertase occurs.

Identity of the enzyme.—Exact identification (in accordance with Pigman's (1946) classification) of the type of carbohydrase studied in the present investigation has not been attempted. For convenience, therefore, we are using the somewhat ambiguous term invertase. That the enzyme is not identical with yeast invertase is indicated by the discrepancies which were formed in the *p*H-activity curves and in the Michaelis constants.

SUMMARY

From indirect evidence it is concluded that the invertise in spores of *M. verrucaria* is located at or near the external surface of the plasma membrane. Rapid synthesis of invertase occurs in the presence of sucrose and yeast extract during germination, synthesis being initiated during the pre-germinative swelling of the spores. When sucrose alone is present, synthesis and growth are much slower. Invertase is not released from intact resting spores. Invertase activity of the spores is not greatly affected by spore age or substrate upon which the spores are produced. The measured activity is equivalent to the actual activity. Assimilation and respiration are apparently at the expense of unhydrolyzed sucrose.

QUARTERMASTER GENERAL LABORATORIES,
PHILADELPHIA, PENNSYLVANIA

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